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Article

The *Drosophila* Ubiquitin-Specific Protease dUSP36/Scny Targets IMD to Prevent Constitutive Immune Signaling

Dominique Thevenon,[1,2,3] Elodie Engel,[1,2,3] Amélie Avet-Rochex,[1,2,3,5] Marie Gottar,[1,2,3,6] Evelyne Bergeret,[1,2,3] Hervé Tricoire,[4] Christelle Benaud,[1,2,3] Jacques Baudier,[1,2,3] Emmanuel Taillebourg,[1,2,3] and Marie-Odile Fauvarque[1,2,3,*]
[1]CEA, DSV, iRTSV, LTS, Institut de Recherches en Technologies et Sciences pour le Vivant, Laboratoire de Transduction du Signal, CEA Grenoble, 38054 Grenoble, France
[2]INSERM U873, 38054 Grenoble, France
[3]Université Joseph Fourier, 38000 Grenoble, France
[4]BFA (EAC 4413) CNRS/Université Paris Diderot, 75013 Paris, France
[5]Present address: CNRS, UMR 5547, 31062 Toulouse, France
[6]Present address: Novartis Institutes for BioMedical Research, 4002 Basel, Switzerland
*Correspondence: marie-odile.fauvarque@cea.fr
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SUMMARY

Ubiquitin proteases remove ubiquitin monomers or polymers to modify the stability or activity of proteins and thereby serve as key regulators of signal transduction. Here, we describe the function of the *Drosophila* ubiquitin-specific protease 36 (dUSP36) in negative regulation of the immune deficiency (IMD) pathway controlled by the IMD protein. Overexpression of catalytically active dUSP36 ubiquitin protease suppresses fly immunity against Gram-negative pathogens. Conversely, silencing *dUsp36* provokes IMD-dependent constitutive activation of IMD-downstream Jun kinase and NF-κB signaling pathways but not of the Toll pathway. This deregulation is lost in axenic flies, indicating that dUSP36 prevents constitutive immune signal activation by commensal bacteria. dUSP36 interacts with IMD and prevents K63-polyubiquitinated IMD accumulation while promoting IMD degradation in vivo. Blocking the proteasome in *dUsp36*-expressing S2 cells increases K48-polyubiquitinated IMD and prevents its degradation. Our findings identify dUSP36 as a repressor whose IMD deubiquitination activity prevents nonspecific activation of innate immune signaling.

INTRODUCTION

Innate immunity relies on immune sensors, such as pathogen-recognition receptors (PRRs), and their ability to immediately activate signals in blood cells and immune organs (Janeway and Medzhitov, 2002). The transcription factors of the nuclear factor-kappa B family (NF-κB) regulate the expression of stress and immune response genes, such as proinflammatory cytokines, antiapoptotic genes, or growth factors (Hoffmann and Baltimore, 2006). Improper activation of these pathways by environmental nonpathogenic microbes or in genetic diseases can lead to serious pathologies such as inflammation and cancers (Karin and Greten, 2005; Pamer, 2007). NF-κB factors act as a surveillance system that is thus permanently repressed through their association in the cytoplasm with I-κB inhibitory molecules. Several pathways stimulate NF-κB, including the Toll-like receptors (TLRs) and the tumor necrosis factor-receptor (TNF-R) pathways. These receptors signal through scaffolding proteins that are subjected to ubiquitination. Depending on their nature, ubiquitin chains can target proteins for degradation or modify their activated status (Terzic et al., 2007). Notably, conjugation of K63-linked polyubiquitin (K63-polyUb) chains to the scaffolding protein RIP1 is required downstream of the TNF-R for the activation of TGF-β-activated kinase 1 (TAK1) and I-κB kinase (IKK) (Ea et al., 2006; Legler et al., 2003). IKK then triggers the phosphorylation of I-κB and its proteasomal degradation through the linkage of K48-polyUb chains by the E3 ubiquitin ligase SCF (Chen et al., 1996), thus releasing free NF-κB for nucleus translocation. A few deubiquitinases (CylD, A20, and Cezanne) have been described to mediate transient inhibition of ubiquitin-dependent activation of the NF-κB pathway (Brummelkamp et al., 2003; Enesa et al., 2008; Kovalenko et al., 2003; Trompouki et al., 2003; Wertz et al., 2004).

In the fruit fly *Drosophila melanogaster*, two conserved immune signaling pathways, Toll and IMD (immune deficiency), are strongly induced in fat-body cells upon infection (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). The Toll pathway is mainly activated by Gram-positive bacteria and fungi and results in the activation of the NF-κB-like *Drosophila* immune factor, which induces a set of antimicrobial peptide genes, including *Drosomycin* (*Drs*) (Lemaitre et al., 1996). The IMD pathway is activated predominantly by Gram-negative bacteria and results in the activation of another set of antibacterial peptide genes, including *Diptericin* (*Dipt*) and *Attacin* (*Att*) (Georgel et al., 2001; Lemaitre et al., 1995; Onfelt Tingvall et al., 2001; Vidal et al., 2001). The IMD pathway is induced by the direct recognition of peptidoglycan via the receptor PGRP-LC/ird7 that interacts with the IMD protein (Choe et al., 2002, 2005; Gottar et al., 2002). IMD is a scaffolding molecule mediating the activation of TAK1, which then drives the activation of two different downstream pathways (Boutros et al., 2002; Georgel et al., 2001; Vidal et al., 2001). On one hand, transient activation of the Jun kinase

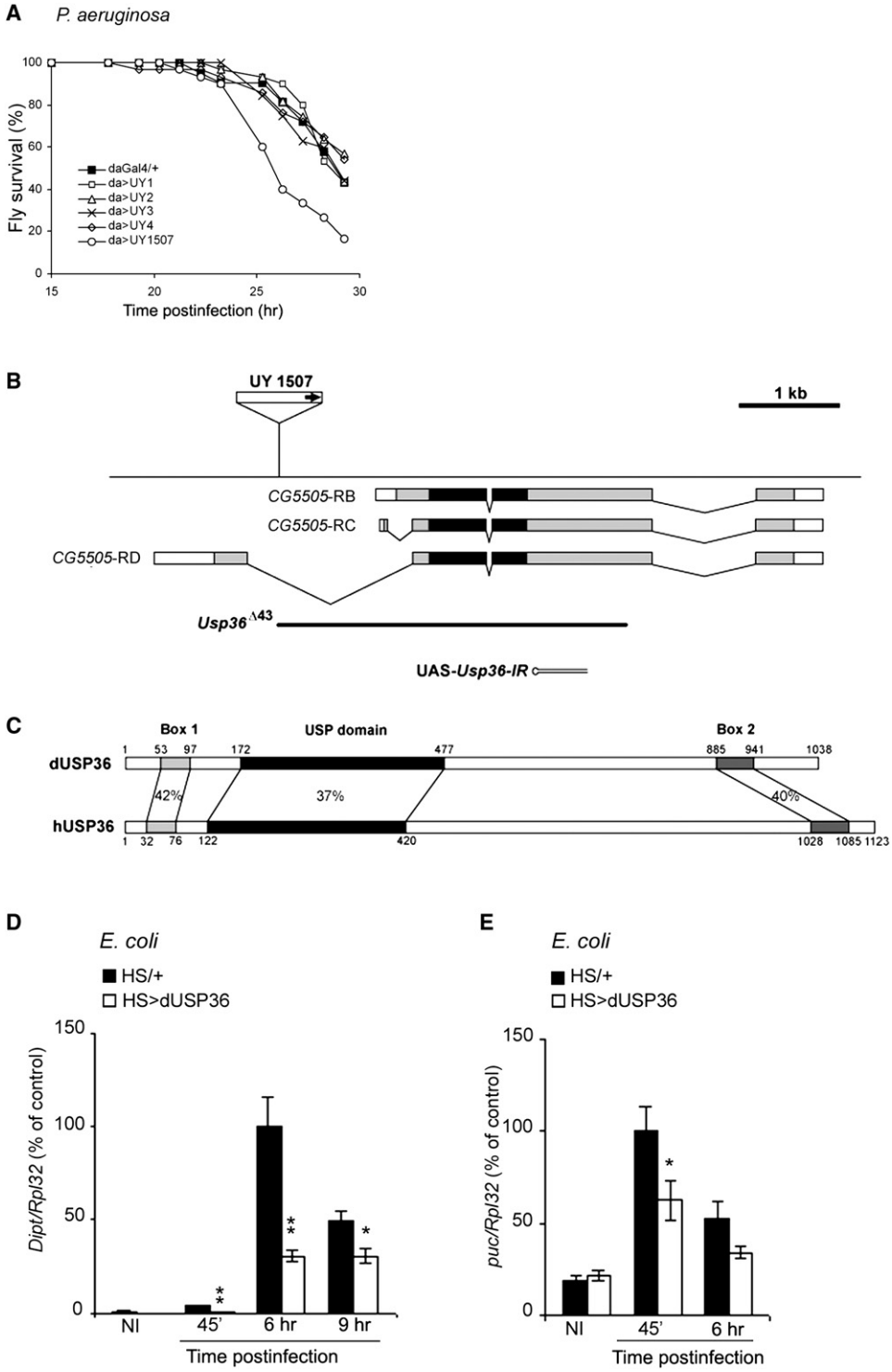

Figure 1. *dUsp36* Overexpression Suppresses Fly Immunity

(A) Forty 5-day-old flies were infected with *P. aeruginosa*, and their survival was followed over 2 days at 20°C. daGal4/UY1507 flies (da>UY1507) presented a sensitive phenotype compared to other UY (#1–4) insertions or to daGal4/+ control flies ($p = < 0.001$, chi-squared test).

(B) Map of UY1507 insertion in *CG5505* transcription unit. Three predicted isoforms (*CG5505-RB*, *-RC*, and *-RD*) are indicated (all isoforms available at http://flybase.org/reports/FBgn0035633.html). The UY1507 insertion is situated upstream of *CG5505-RB* and *-RC*. Grey box corresponds to coding sequence, and black box indicates USP catalytic domain. Black line indicates the extension of the $dUsp36^{\Delta 43}$ deficiency. Double-strand line indicates location of inverted repeats used to silence *dUsp36* in vivo (P{UAS-*Usp36-IR*}).

(C) Percentage of sequence identity between *CG5505-RC* encoded protein (dUSP36) and hUSP36 predicted protein. In addition to the USP domain (black box, 37% identity), the two sequences align over two other conserved domains (boxes 1 and 2, showing 42% and 40% identity, respectively).

(JNK) pathway induces genes encoding proteins required for wound repair and the downregulatory phosphatase *puckered* (*puc*). On the other hand, TAK1 phosphorylates Kenny (KEY), the *Drosophila* IKKγ, which then phosphorylates the NF-κB-like factor Relish (Rel) (Kleino et al., 2005; Silverman et al., 2000). As in mammalian cells, a set of conserved ubiquitin ligases regulates the *Drosophila* Toll and IMD pathways (Chiu et al., 2005; Khush et al., 2002; Tsuda et al., 2005; Zhou et al., 2005). The orthologous *Drosophila* CylD is the sole deubiquitinase whose function has been assessed in *Drosophila* immune response: it specifically binds KEY, and its overexpression downregulates antimicrobial peptide gene expression (Tsichritzis et al., 2007). At the present date, however, deubiquitinases that would act on upstream elements of the IMD pathway still remain to be identified.

In this study, we describe the function of *Drosophila* ubiquitin-specific protease 36 (dUSP6) in the inhibition of immune signaling. Independent studies showed that dUSP36 (Scrawny [Scny]) is required for stem cell maintenance and histone H2B deubiquitination (Buszczak et al., 2009) and is also involved in apoptosis regulation during development (Emperor's thumb [ET]) (Ribaya et al., 2009). Since we show that *dUsp36/scny/et* is homologous to human *USP36* (*hUSP36*) (Kim et al., 2004, 2005; Quesada et al., 2004), we use the nomenclature *dUsp36* to indicate its inclusion in the USP gene family (Nijman et al., 2005). We demonstrate that dUSP36 is required to prevent IMD activation both in *Drosophila* S2 cells and in adult flies. Overexpression of dUSP36 suppresses *Drosophila* immunity and antimicrobial peptide gene expression via its catalytic activity. Moreover, we created a null *dUsp36* allele ($dUsp36^{\Delta 43}$), and using conditional rescue during development or conditional expression of a silencing transgene in adult gut and fat-body cells, we observed that loss of *dUsp36* expression induces constitutive deregulation of the IMD and JNK pathways but not of the Toll pathway. IMD activation in *dUsp36*-silenced gut was lost in axenic flies or in IMD-silenced flies, indicating that dUSP36 prevents IMD activation by environmental bacteria and is acting upstream of IMD. Indeed, dUSP36 interacts with IMD in vivo, and its expression, but not that of the catalytically dead mutant, decreased the amount of both endogenous IMD and K63-ubiquitinated forms. Similar results were obtained in S2 cells where, in addition, blocking the proteasome in *dUsp36*-overexpressing cells increased K48-ubiquitinated IMD and prevented its degradation. Therefore, dUSP36 likely prevents signal transduction by removing K63-polyUb and indirectly promoting the linkage of K48-polyUb.

RESULTS

A P{UAS} Insertion in *dUsp36* Suppresses Fly Immunity to Gram-Negative Pathogens

In a gene candidate approach for genes involved in the *Drosophila* immune response (Monnier et al., 2002) (see Experimental Procedures), we selected the insertion P{MaeUAS.6.11}UY1507, which caused sensitivity to the Gram-negative virulent strain *Pseudomonas aeruginosa* (Figure 1A). UY1507 is inserted in the *CG5505* gene 5′ to -RB and -RC isoforms (Figure 1B). It drives the expression of two *CG5505* transcripts, which likely correspond to these two isoforms and further provoked a marked reduction of *Dipt* induction following bacterial infection (Figure S1A). Quantification of *CG5505* mRNAs showed an 8-fold increase in daGal4/UY1507 flies (Figure S1B). Blast analysis revealed two close homologous proteins in the human genome: hUSP36 and hUSP42. The longest isoform, *CG5505-RD* (Figure 1B), encodes a predicted protein possessing the highest homology with hUSP36. Indeed, in addition to the USP catalytic domain (black box), two other conserved domains presenting 40% and 42% identity with hUSP36 were detected in the N-terminal and C-terminal parts (Figure 1C, boxes 1 and 2). These domains are not present in hUSP42 and do not share any similarities with previously identified domains. We thus conclude that *hUSP36* is the closest homolog to *CG5505*.

To assess whether the immune phenotype observed with the insertion would be due to *dUsp36* overexpression, we constructed P{UAS-*dUsp36*} transgenic flies expressing the *CG5505-RB* transcript. Transgene expression was induced through the heat shock (HS) driver line, HSGal4, and resulted in high levels of *dUsp36* transcripts (see below). Overexpressing *dUsp36* decreased the induction of both the IMD target gene *Dipt* at 45 min, 6 hr, and 9 hr postinfection (Figure 1D) and the JNK target gene *puc* at 45 min postinfection (Figure 1E). Moreover, as observed with the UY1507 insertion, overexpressing *dUsp36* provoked fly sensitivity to *P. aeruginosa* and *E. cloacae* but not to nonpathogenic bacteria *Agrobacterium tumefaciens* (Figures S1C–S1E) or *Escherichia coli* (not shown). Overexpressing *dUsp36* thus prevents the activation of the IMD pathway and causes flies' sensitivity to Gram-negative pathogens.

dUSP36 Suppresses Fly Immunity via Its Catalytic Activity

Several intermediates of the IMD pathway might be activated through the linkage of ubiquitin monomers or polymers. This raises the possibility that dUSP36 is a deubiquitinating enzyme regulating the IMD pathway through its catalytic activity. We showed that the catalytic domain of dUSP36, like its human counterpart hUSP36 (Kim et al., 2004; Quesada et al., 2004), hydrolyzed a Ub-β-gal substrate when coexpressed in bacteria (Figure S2A) and that both human and *Drosophila* proteins also digested polyUb isopeptide bound in vitro (Figures S2B and S2C). Mutating the two catalytic conserved residues (C*H*) resulted in a total loss of activity (Figures S2A and S2B). To test for the requirement of dUSP36 catalytic activity in the negative regulation of the IMD pathway in vivo, we constructed P{UAS-*dUsp36C*H**}-transgenic flies expressing the catalytically dead mutant. HS-driven expression of *UAS-dUsp36C*H** induced amounts of transcript and protein similar to the wild-type form (Figures S3A and S3B). Unlike dUSP36, overexpressing dUSP36C*H* did not inhibit *Dipt* induction following *E. coli* infection (Figure 2A). Similarly, overexpressing dUSP36, but not the

(D and E) Quantitative analysis of *Dipt* (D) and *puc* (E) mRNAs by quantitative RT-PCR in noninfected (NI) flies or at 45 min (45′), 6 hr, or 9 hr postinfection with *E. coli*. Results are expressed as percent of the maximal induction level observed in dUSP36-expressing flies (HS>dUSP36) compared to control HS/+ flies (100%). Values represent the mean of three technical replicates. Error bars represent SD. One representative experiment out of three biological independent experiments is presented. Significant difference of $p < 0.05$ (*) or of $p < 0.01$ (**) compared to HS/+ control flies (Student's t test).

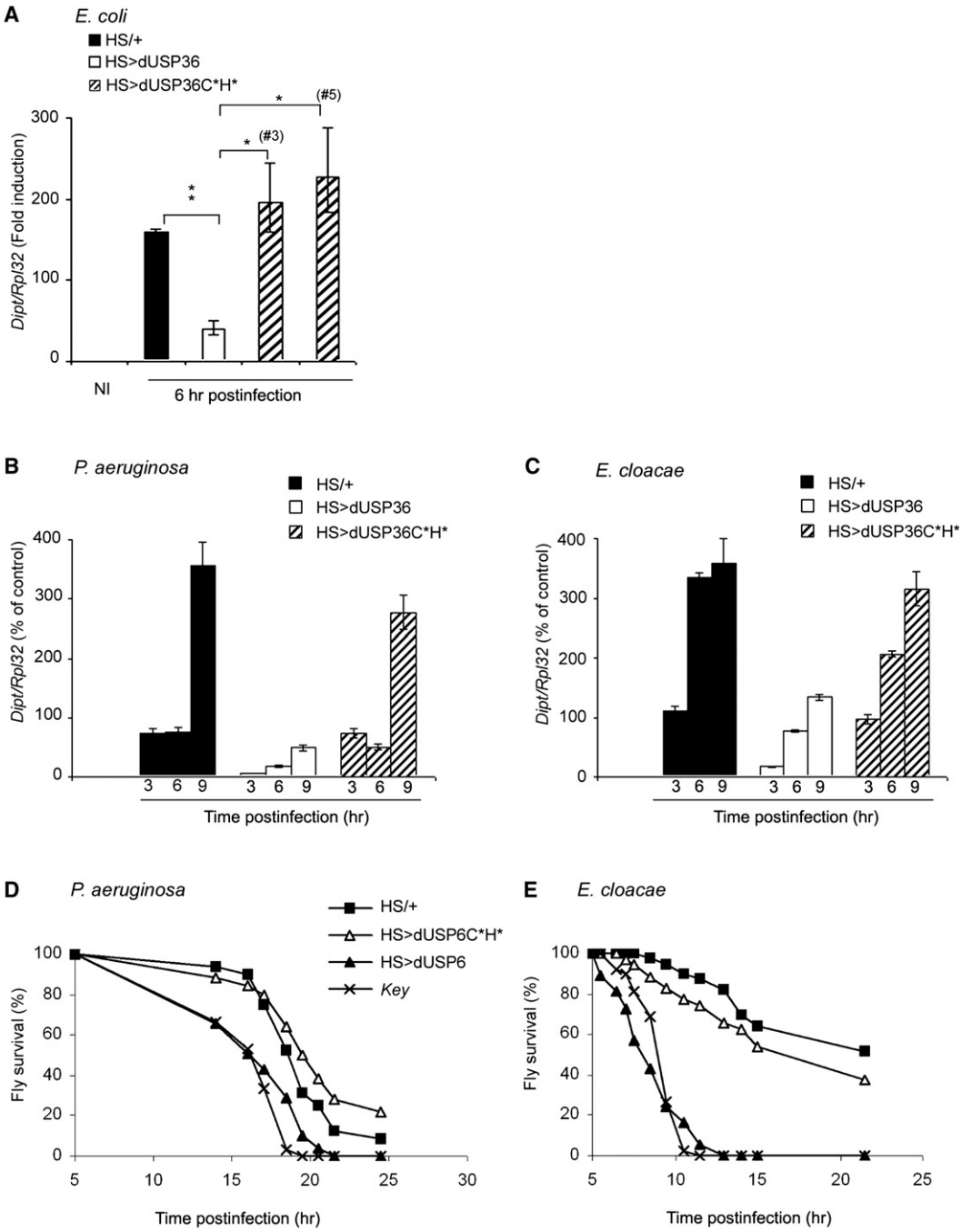

Figure 2. dUSP36 Suppresses Fly Immunity through Its Catalytic Activity

(A) Analysis of *Dipt* mRNAs by quantitative RT-PCR in noninfected (NI) flies or at 6 hr postinfection with *E. coli*. Results are expressed as the fold induction level compared to noninfected HS/+ flies (NI). HS-driven expression of *dUsp36* (in UAS-*dUsp36*/Y;HSGal4/+ flies) strongly reduces *Dipt* induction following infection (HS>dUSP36). In contrast, HS-driven expression of the mutant *dUsp36C*H** has no effect on *Dipt* expression level, as observed in two independent transgenic lines (HS>dUSP36C*H* #3 and #5).

(B and C) Similar analysis of *Dipt* mRNAs at 3, 6, and 9 hr postinfection with either *P. aeruginosa* (B) or *E. cloacae* (C) showed that overexpressing dUSP36, but not dUSP36C*H*, impaired *Dipt* induction. Values in (A)–(C) represent the mean of three technical replicates. Error bars represent SD. One representative experiment out of three biological independent experiments is presented. Significant differences of $p < 0.01$ (**) or $p < 0.05$ (*).

(D and E) Forty 5-day-old flies were infected with *P. aeruginosa* (D) or *E. cloacae* (E) 16 hr following HS treatment, and their survival kinetics were followed over 2 days. The *key* mutant is used as a Gram-negative sensitive control. HS-driven expression of the *dUsp36* transgene (HS>dUSP36), but not of *dUsp36C*H** (HS>dUSP36C*H*), results in fly sensitivity to both pathogens as compared to HSGal4/+ control flies ($p < 0.01$, chi-squared test).

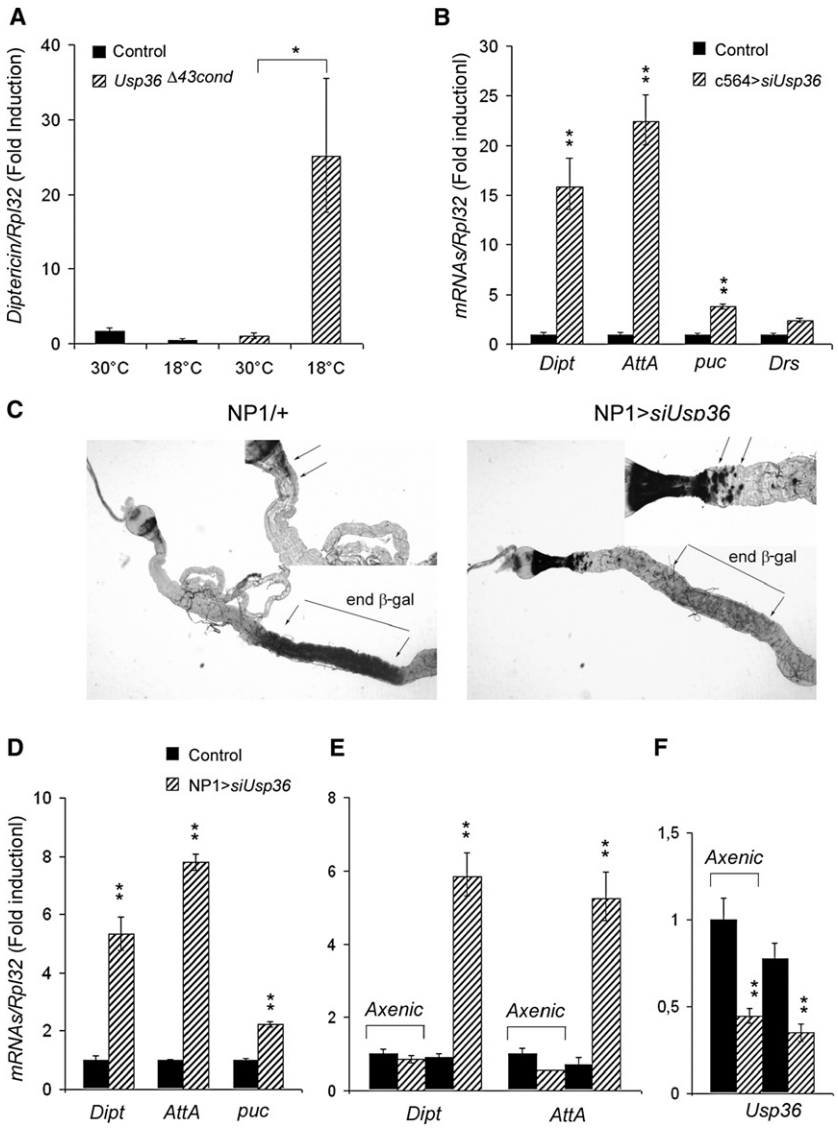

Figure 3. dUSP36 Is Required to Prevent Constitutive Induction of the IMD Pathway In Vivo

(A) Analysis of *Dipt*, *AttA*, *puc*, and *Drs* mRNAs by quantitative RT-PCR. Control flies issued from perfect excision of UY1507 (Control) or UAS-*dUsp36*/+; $Gal80^{ts}$/+; daGal4,$dUsp36^{\Delta 43}$/$dUsp36^{\Delta 43}$ flies ($dUsp36^{\Delta 43cond}$) were raised at 30°C during development, and then a set of flies was placed 4 days at 18°C at the adult stage—preventing UAS-*dUsp36* expression—and was compared to control siblings maintained at 30°C. Temperature change has no effect on *Dipt* expression in control flies placed at either 18°C or 30°C. Significant difference between $dUsp36^{\Delta 43cond}$ flies raised at 30°C compared to 18°C was $p < 0.05$ (*) (Student's t test).

(B) Fat-body-directed silencing of *dUsp36* was induced at the adult stage by raising c564Gal4/$Gal80^{ts}$, UAS-*dUsp36-IR* flies at 18°C during development and rearing adults for 4 days at 30°C (*siUsp36*, hatched bars). Results are presented as the fold induction of each antimicrobial peptide-encoding gene mRNA (*Dipt*, *AttA*, or *Drs*) or the phosphatase-encoding gene *puc* in *dUsp36* fat-body-silenced flies compared to control flies (black bars). Significant difference with control flies was $p < 0.01$ (**).

(C-F) Gut-directed silencing of *dUsp36* was induced with the NP1Gal4 driver line using protocol similar to that in (B). Ectopic activation of a *Dipt-lacZ* reporter transgene is observed only in the anterior midgut (C, arrows on the larger view shown on the top right of each picture). Area of endogenous β-gal is indicated (end β-gal). Induction of *Dipt*, *AttA* and *puc*, *or dUsp36* (D–F) was monitored by quantitative RT-PCR on isolated adult guts. Guts from flies raised in normal conditions are shown in (D) and guts isolated from axenic flies or from their control siblings raised in similar conditions in (E) and (F). Values in (A), (B), and (D)–(F) represent the mean of three technical replicates. Error bars represent SD. One representative experiment out of three biological independent experiments is presented. Significant difference with control flies of $p < 0.01$ (**) or $p < 0.05$ (*).

catalytically inactive form, inhibited the induction of *Dipt* following infection by the pathogenic bacteria *P. aeruginosa* and *E. cloacae* (Figures 2B and 2C). Decreased antimicrobial peptide expression is associated with increased fly sensitivity to these Gram-negative pathogens when overexpressing dUSP36, but not the catalytically inactive form (Figures 2D and 2E). Our experiments demonstrate that dUSP36 negatively regulates the IMD pathway in vivo and suppresses fly immunity through its catalytic activity. Thus, we conclude that the deubiquitinating activity of dUSP36 is essential for inhibition of the IMD pathway.

dUSP36 Prevents Constitutive Activation of IMD and JNK Pathways

To decipher the in vivo function of dUSP36, we created the null mutation $dUsp36^{\Delta 43}$ (Figure 1A). $dUsp36^{\Delta 43}$ homozygotes were lethal at early larval stage (E.T., unpublished data). Lethality of $dUsp36^{\Delta 43}$ mutants was rescued by expressing the UAS-*dUsp36* construct under the control of the ubiquitous daGal4 driver, allowing for the recovery of viable adult flies. This indicates that loss of *dUsp36* is responsible for lethality of $dUsp36^{\Delta 43}$ homozygotes. To elucidate the requirement of dUSP36 in the immune response at the adult stage, the lethality of $dUsp36^{\Delta 43}$ mutants was rescued by expressing the UAS-*dUsp36* transgene during development under the control of the daGal4 driver, itself conditionally inactivated at the adult stage by the Gal80 thermosensitive ($Gal80^{ts}$) inhibitor of Gal4 ($dUsp36^{\Delta 43cond}$) (McGuire et al., 2003). A 20-fold increase of *Dipt* expression was observed in $dUsp36^{\Delta 43cond}$ flies placed 4 days at 18°C compared to flies of the same genotype maintained at 30°C (Figure 3A). This increase was not observed in control flies raised at 18°C or 30°C (Figure 3A). Loss of *dUsp36* expression at the adult stage thus results in a constitutive activation of the IMD pathway.

We then constructed P{UAS-*dUsp36-IR*}-transgenic lines expressing inverted repeat cDNA sequences to inactivate *dUsp36* expression in vivo (Figure 1A). Ubiquitous expression of this construct resulted in early larval lethality, thus suggesting that it efficiently silenced *dUsp36*, as confirmed by monitoring dUSP36 protein level from total fly extracts (Figure S3C).

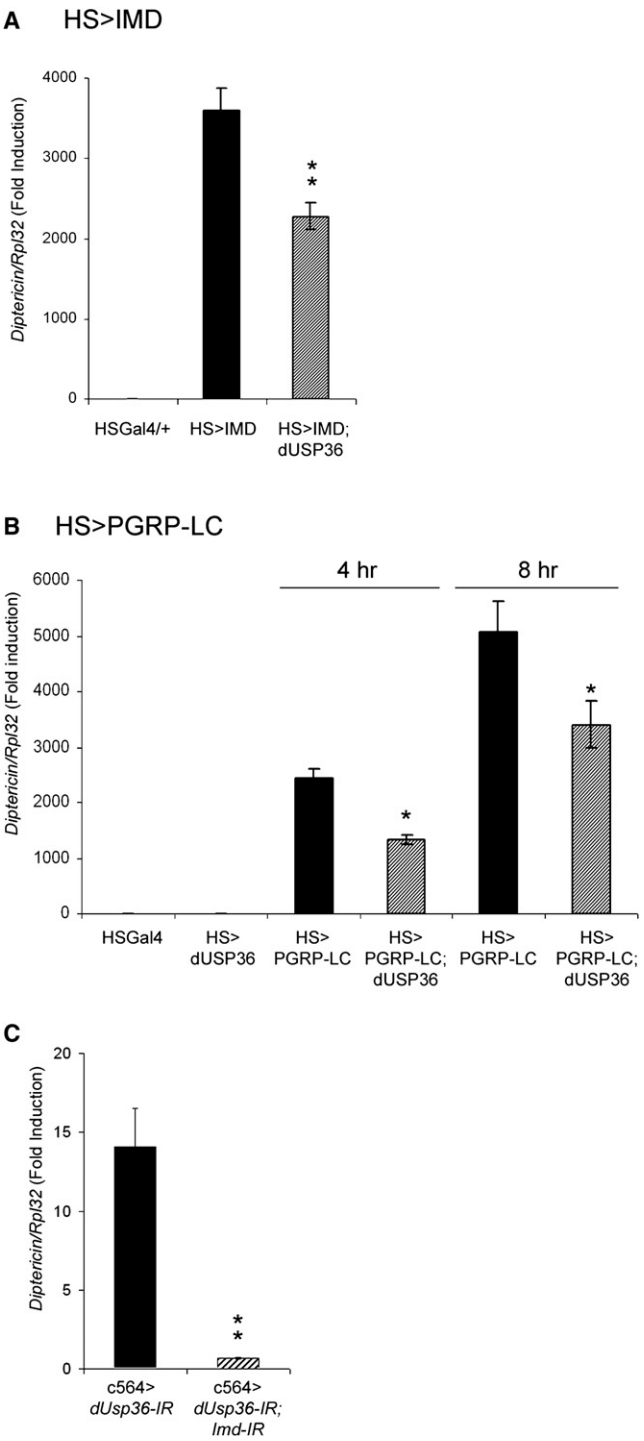

Figure 4. dUSP36 Function Depends on IMD In Vivo

(A–C) Analysis of *Dipt* mRNAs by quantitative RT-PCR. *Dipt* expression was analyzed in flies overexpressing IMD alone (HS>IMD) or in combination with dUSP36 (HS>IMD;dUSP36) at 16 hr following HS treatment (A). Flies were submitted to a mild HS for 1 hr at 37°C, and *Dipt* expression was analyzed at 4 and 8 hr following HS (B). *Dipt* expression was induced by overexpressing PGRP-LC alone (HS>PGRP-LC) or with dUSP36 (HS>dUSP36;PGRP-LC). Conditional silencing in adult fat body of *dUsp36* (c564>*dUsp36-IR*) (genotype: c564Gal4/Gal80^{ts},*UAS-dUsp36-IR* flies) or of *dUsp36* and *Imd* jointly (c564>*dUsp36-IR,Imd-IR*) (genotype: c564gal4/Gal80^{ts},UAS-*dUsp36-IR*; UAS-*Imd-IR*/+) in noninfected flies (NI) is shown in (C). To achieve conditional silencing, flies were raised at 18°C during development and 30°C for 4 days before analysis of *Dipt* transcripts. Results are expressed as the fold induction level compared to c564Gal4/+ flies raised in similar conditions. The constitutive deregulation of *Dipt* gene expression caused by silencing *dUsp36* in c564>*dUsp36-IR* is totally prevented by cosilencing *Imd* (c564>*dUsp36-IR,Imd-IR*). Values represent the mean of three technical replicates. Error bars represent SD. One representative experiment out of three biological independent experiments is presented. Significant differences with control are indicated: $p < 0.05$ and $p < 0.01$ (**) (Student's t test).

dUsp36 silencing in fat-body cells using the c564Gal4 driver line (Hrdlicka et al., 2002) also resulted in larval lethality, indicating that dUSP36 function in the fat body is vital during development. Expression of the UAS-*dUsp36-IR* transgene was then conditionally controlled with the Gal80^{ts} inhibitor 48 hr after pupal eclosion by raising adults for 4 days at 30°C. Silencing *dUsp36* in the adult fat body of noninfected flies resulted in *Dipt*, *AttA*, and *puc* induction (Figure 3B). The relatively low induction level of *puc* mRNA (3.7-fold) is significant compared to the 4- to 5-fold induction observed in infected flies at 45 min postinfection with *E. coli* (Figure 1E). In contrast, the low level of induction (2-fold) observed in the case of *Drs* is not significant compared to the 40-fold induction observed in *M. luteus*-infected flies at 48 hr postinfection (data not shown) (Figure 3B). These results indicate that IMD and JNK pathways are specifically activated in *dUsp36*-silenced fat-body cells.

In addition to fat-body cells, the gut is another immune-competent tissue that can activate the IMD pathway upon oral infection (Buchon et al., 2009). *dUsp36* conditional silencing with the gut-specific driver line NP1Gal4 carrying the *dipt-lacZ* reporter gene resulted in ectopic activity of β-galactosidase in the anterior midgut (Figure 3C). Deregulation of *Dipt*, *AttA*, and *puc* was further quantified from *dUsp36*-silenced guts and reached, respectively, 5-, 8-, and 2-fold induction compared to control guts (Figure 3D). The presence of environmental bacteria may be responsible for IMD pathway constitutive activation in *dUsp36*-silenced gut. Axenic flies without commensal bacteria were created by sterilizing eggs and raising flies in sterile conditions. Interestingly, no deregulation of *Dipt* and *AttA* was observed in *dUsp36*-silenced axenic fly guts compared to nonsterilized siblings raised in similar conditions (Figure 3E). More than 2-fold decreased expression of *dUsp36* was monitored in corresponding guts (Figure 3F). dUSP36 is thus required to permanently prevent activation of IMD-dependent pathway in response to environmental bacteria.

dUSP36 In Vivo Function Depends on IMD

Overexpressing upstream components of the IMD pathway such as PGRP-LC, IMD, or TAK1 results in antimicrobial peptide gene induction in the absence of immune challenge (Choe et al., 2002; Georgel et al., 2001; Vidal et al., 2001). To assess at which level dUSP36 acts on the IMD pathway, dUSP36 was coexpressed with PGRP-LC, IMD, or TAK1 in double-transgenic lines, and the activation of *Dipt* was analyzed by quantitative RT-PCR. HS-driven expression of dUSP36 with IMD decreased the level of *Dipt* activation observed with IMD alone (Figure 4A). Because of the very poor viability of PGRP-LC- and TAK1- overexpressing flies, we used a milder heat-shock protocol (1 hr at 37°C). In these conditions, overexpressing dUSP36 reduced the level of

Dipt expression induced by overexpressing PGRP-LC (Figure 4B), but not that induced by overexpressing TAK1 (Figure S4), suggesting that dUSP36 is acting upstream or at the level of IMD. To further test whether deregulation of the IMD pathway in *dUsp36*-silenced fat-body cells depends on the presence of IMD, both genes were simultaneously silenced at the adult stage in fat-body cells. We first verified that the UAS-*Imd-IR* transgene used in our study efficiently silenced *Imd*. Indeed, compared to control flies, silencing *Imd* in fat-body cells prevented *Dipt* expression following *E. coli* infection and provoked a marked reduction of detectable endogenous IMD protein (Figure S5). We then conditionally silenced *dUsp36*, either alone or in combination with *Imd*, in the adult fat body using the Gal80^{ts} system described above. As previously mentioned, silencing *dUsp36* in the adult fat body resulted in a constitutive deregulation of the IMD pathway, whereas no deregulation of *Dipt* gene expression occurred in double *dUsp36*- and *Imd*-silenced flies (Figure 4C). This last result indicates that deregulation of the IMD pathway mediated by silencing *dUsp36* strictly requires IMD. Importantly, the lethality of *dUsp36* null mutants is not rescued by mutations in the *TAK1*, *Dredd*, *Diap2*, *key*, or *Imd* genes. This indicates that constitutive induction of the IMD pathway is not the cause for the lethality of *dUsp36* null mutants.

dUSP36 Interacts with IMD

The analysis of the in vivo function of dUSP36 suggests that it permanently prevents signal transduction through IMD (Choe et al., 2005). We thus used a coimmunoprecipitation (coIP) assay in transfected S2 cells to investigate the ability of dUSP36 to interact with IMD or PGRP-LC (or KEY as a negative control). Remarkably, IMD, but not PGRP-LC nor KEY, coimmunoprecipitated dUSP36 (Figure 5A). CoIP was further observed in both directions between dUSP36 and the full-length IMD and between dUSP36 and IMD N-terminal part (Figure 5B), but not with IMD C-terminal part (data not shown). To further map the interaction domain of dUSP36 with IMD, we performed a GST pull-down assay with two truncated forms of the dUSP36 protein. In this assay, the C-terminal part but not the N-terminal part of dUSP36 did specifically retain the recombinant IMD protein (Figure 5C). Finally, the interaction between dUSP36 and IMD was confirmed in vivo with proteins coimmunoprecipitated from fly extracts (Figure 5D).

In a previous study, dUSP36/Scny expression was detected mainly in the nucleus, where it deubiquitinates H2B (Buszczak et al., 2009). Since our results indicate that dUSP36 acts at the level of IMD, which is thought to act in the cytoplasm, we suggested that different isoforms of dUSP36/Scny would display different subcellular localization. To assess this hypothesis, the Myc-tagged *CG5505*-RB isoform used in this study was expressed in S2 cells together with IMD-V5. Corresponding proteins were detected in the cell cytoplasm (Figure S6).

dUSP36 Decreases IMD and K63-PolyUb IMD Levels and Promotes IMD Proteasomal-Dependent Degradation

In mammalian cells, K63-linked polyubiquitin chains on RIP1 are recognized by the ubiquitin-binding domain of TAB2, which recruits TAK1 (Ea et al., 2006; Legler et al., 2003; Newton et al., 2008). The deubiquitinase A20 downregulates the signal by hydrolyzing K63-linked chains and catalyzing the linkage of K48-linked chains, thus promoting RIP1 degradation. In *Drosophila*, IMD has scaffolding function similar to RIP1. We observed that IMD is constitutively ubiquitinated by K63-polyUb chains in vivo (Figure 6A) or when overexpressed in *Drosophila* S2-transfected cells (Figure 6B). Expressing dUSP36 but not the catalytically mutant form dramatically decreased K63-ubiquitinated IMD and also lowered the amount of endogenous full-length IMD (Figure 6A). Similar results were obtained in S2 cells: coexpressing dUSP36 with IMD reduced the amount of K63-ubiquitinated forms of IMD when compared to control cells or to cells expressing the catalytically inactive mutant dUSP36C*H* (Figure 6B). In addition, expressing dUSP36 also decreased the total amount of IMD detected in the cell lysate, thus indicating that dUSP36 promotes IMD degradation (Figure 6B). Blocking the proteasome by treating cells with MG132 restored a normal amount of IMD protein in dUSP36-transfected cells (Figure 6B). Moreover, K48-ubiquitinated IMD was detected in MG132-treated cells, and dUSP36 expression in these cells increased the amount of K48-ubiquitinated forms of IMD (Figure 6B). All together, these results suggest that dUSP36 hydrolyzes the activating K63-polyUb chains on IMD. Removal of K63-polyUb chains may then favor K48-polyUb linkage and subsequent degradation of IMD.

DISCUSSION

Our work identifies dUSP36 as a deubiquitinating enzyme that negatively regulates the IMD-dependent signaling pathway in *Drosophila*. We show that dUSP36 is required to prevent constitutive activation of the IMD pathway in the absence of immune challenge. Indeed, silencing *dUsp36* either in the whole organism or only in the adult fat body or gut induces a constitutive expression of IMD-dependent antimicrobial peptide-encoding genes. We further showed that deregulation of the pathway depends on the presence of environmental bacteria, as no deregulation was observed in guts isolated from axenic flies. This indicates that dUSP36 is needed to prevent IMD pathway activation by environmental bacteria and is required for immune tolerance, as previously shown for PIMS, another negative regulator of the IMD pathway (Lhocine et al., 2008). Deregulation of the pathway observed in *dUsp36*-silenced fat body may similarly occur via bacterial peptidoglycan release crossing the intestinal barrier. The JNK pathway is also deregulated by *dUsp36* silencing, as shown by elevated expression of the JNK target gene *puc*. Deregulation of the JNK pathway might be a consequence of IMD activation, since JNK can be activated through TAK1, itself activated by IMD (Vidal et al., 2001). In contrast, the expression of *Drs*, a target gene of the Toll pathway, was not significantly modified. These results indicate that dUSP36 requirement in the negative regulation of innate immune signals is specific for the IMD pathway.

Since *dUsp36* silencing results in constitutive activation of the IMD pathway, it could have been expected that *dUsp36* mutant flies would resist better to bacterial infections. In fact, we observed the opposite phenotype: *dUsp36*-silenced flies are more sensitive to infections (data not shown). Deregulation of immune signals has been previously shown to be detrimental and to induce fly sensitivity to microbial infections (Bischoff

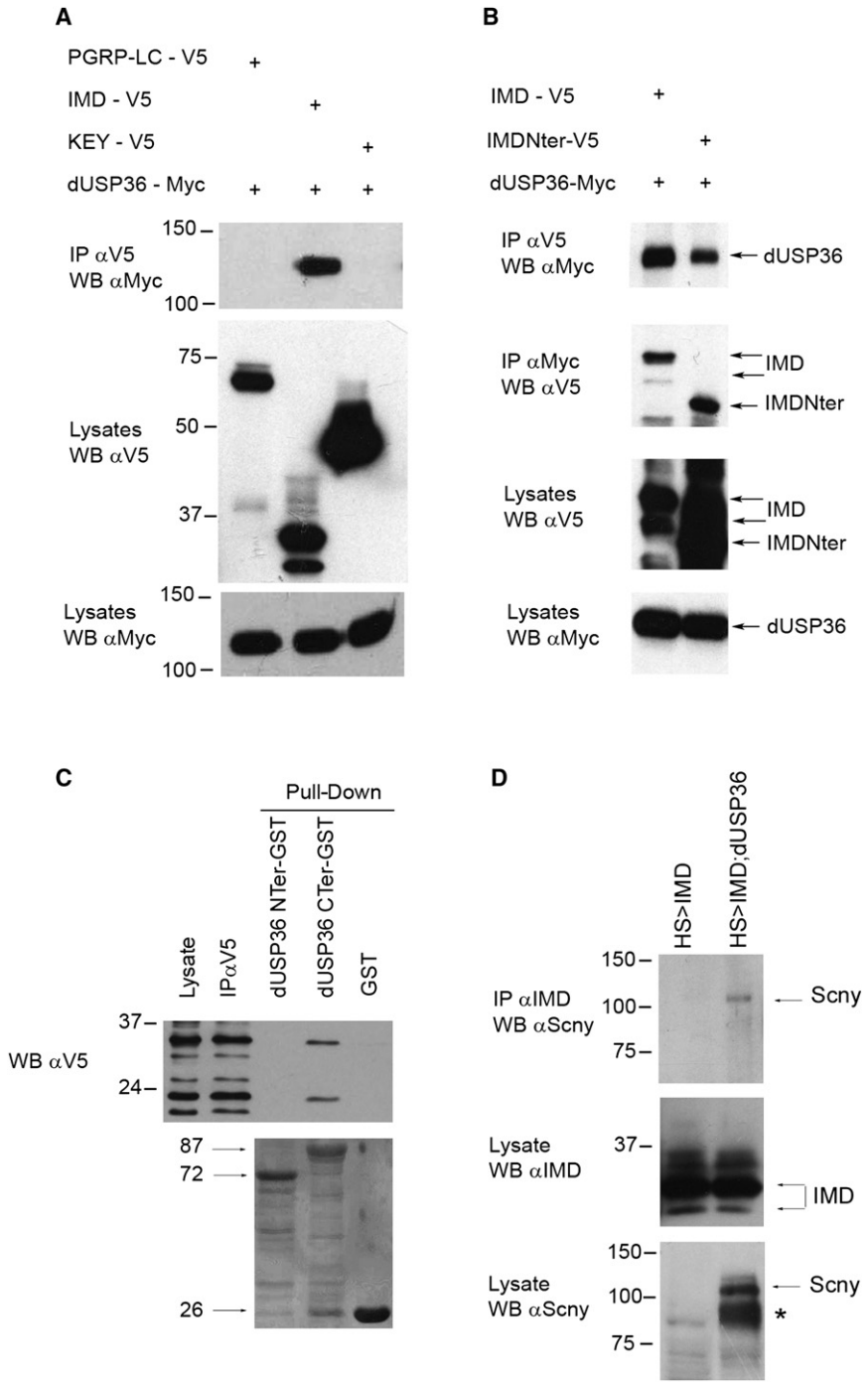

Figure 5. dUSP36 Interacts with IMD

(A) *Drosophila* S2 cells were cotransfected with the expression constructs encoding for PGRP-LC-V5, IMD-V5, or KEY-V5 and dUSP36-Myc. Cell extracts were coimmunoprecipitated with anti-V5 antibodies and analyzed by western blot with anti-Myc and anti-V5 antibodies (IPV5, αV5: not shown). Presence of recombinant proteins in the input was assessed by western blot of cell extracts with anti-V5 and anti-Myc antibodies. The IMD protein migrates as four major bands ranging in size from 20 to 35 kDa. The two higher bands, which correspond, respectively, to the full-length (35 kDa) and a cleaved form at 30 kDa (N. Silverman, personal communication), are shown.

(B) Similar experiments were performed in cells expressing dUSP36-Myc with either IMD-V5 or its N-terminal part (IMDNter-V5). Cell extracts were immunoprecipitated with either anti-V5 antibodies (IPV5, WBαMyc) or anti-Myc antibodies (IPαMyc, WBαV5).

(C) S2 cells were transfected with the expression construct encoding IMD-V5 and lysed after 48 hr. Cells lysates were subjected to either immunoprecipitation with an antibody against V5 or to GST pull-down assays using GST fusion proteins with either the dUSP36[41–457] N-terminal part (dUSP36 Nter) or the dUSP36[418–968] C-terminal part (dUSP36 Cter) or with GST alone (GST). Lysates were colored with Ponceau red to visualize GST fusion proteins in the input (bottom part).

(D) Protein extracts were prepared from flies overexpressing IMD alone (HS>IMD) or with dUSP36 (HS>IMD; dUSP36) at 16 hr following HS. IMD was immunoprecipitated from fly extracts with antibodies directed against IMD (gift from J.M. Reichhart) and analyzed with anti-Scny (dUSP36) (Buszczak et al., 2009). Anti-Scny antibodies revealed a unique band at the expected size (130 kDa) showing that dUSP36 and IMD form a complex in vivo. Fly extracts were revealed with anti-IMD and anti-Scny to show presence of corresponding proteins in the fly extracts (*, unspecific band).

et al., 2006; Gordon et al., 2008; Maillet et al., 2008). Moreover, independent studies showed pleiotropic functions of dUSP36 that might interfere with fly survival upon infection (Buszczak et al., 2009; Ribaya et al., 2009). Since a few deubiquitinases exist in eukaryotic genomes (around 25 in *Drosophila melanogaster*), it is expected that each enzyme would have several substrates. Specific interactions with other proteins would determine substrate specificity. It is also important to note that, even if hydrolyzing both K48- and K63-linked ubiquitin chains in vitro, a deubiquitinating enzyme may specifically remove K63-linked chains on a given substrate in vivo. This seems to be the case for dUSP36 (this study) and has previously been observed with A20 (Wertz et al., 2004).

Overexpressing dUSP36 but not a catalytically inactive form inhibits the activation of the IMD pathway, resulting in increased fly sensitivity to Gram-negative pathogens. This indicates that dUSP36 negatively regulates the IMD pathway through its catalytic activity. We further provide evidence that dUSP36 acts on the IMD pathway at the level of IMD. First, overexpressing dUSP36 reduces the constitutive activation of the pathway provoked by overexpressing either PGRP-LC or IMD, but not that produced by overexpressing TAK1. Second, constitutive activation of the IMD pathway induced by dUSP36 silencing strictly depends on the presence of IMD. Finally, dUSP36 associates with the N-terminal part of IMD through its C-terminal domain, and its expression, but not the

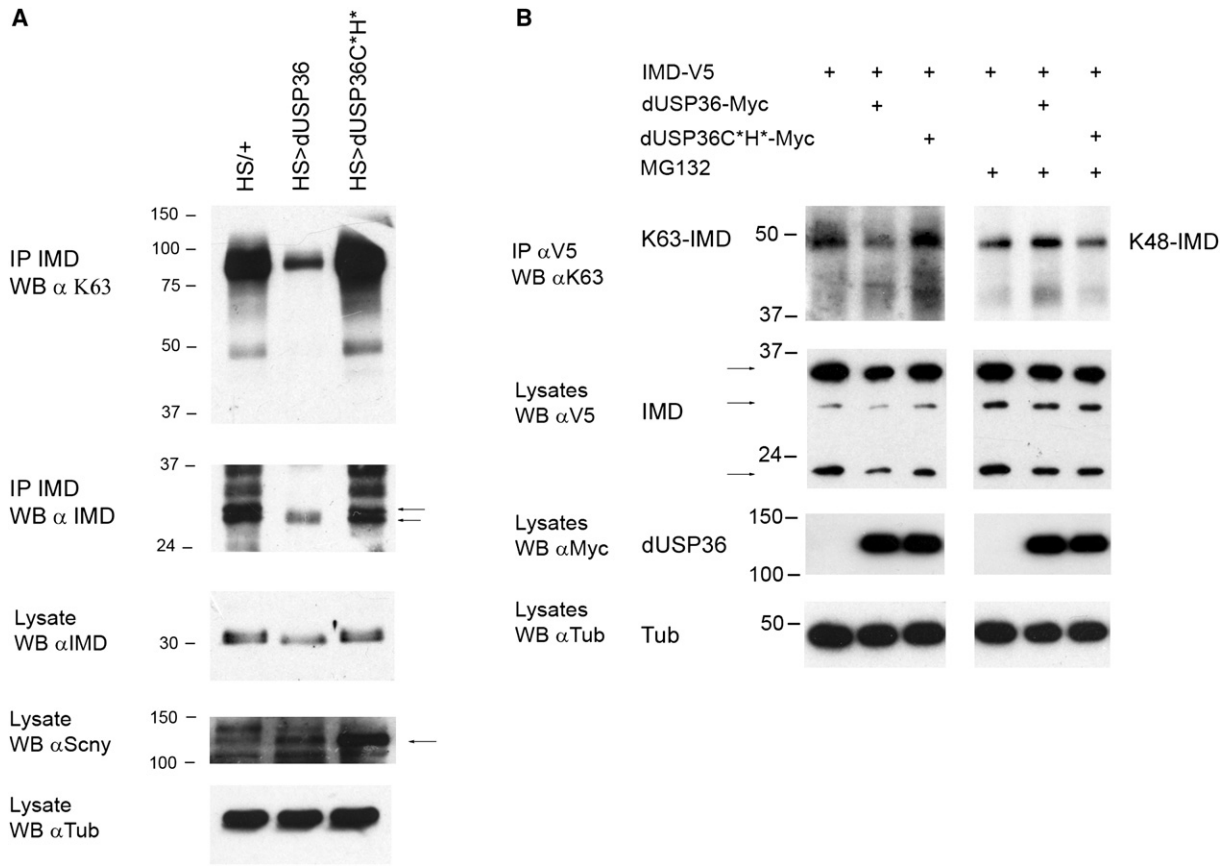

Figure 6. dUSP36 Decreases K63-Ubiquitinated IMD while Promoting Its Proteasomal-Dependent Degradation

(A) Protein extracts were prepared from control flies (HS/+) or flies expressing either dUSP36 (HS>USP36) or the catalytically dead form (HS>USP36C*H*) at 16 hr following HS. IMD was immunoprecipitated from fly extracts and analyzed with anti-K63 antibodies (Newton et al., 2008) (IPV5 αK63) or anti-Imd (IPV5 αV5). Fly extracts were revealed with anti-IMD (Lysates αIMD) and anti-Scny (Lysates αScny) to detect the corresponding proteins in the input (*, unspecific band). IPV5 αK63 revealed bands ranging from 31 to 50 kDa showing several K63-ubiquitinated IMD species.

(B) *Drosophila* S2 cells were transfected with IMD-V5 alone or with either wild-type dUSP36-Myc or mutated dUSP36C*H*-Myc. Cell extracts were immunoprecipitated with anti-V5 antibodies and analyzed with anti-K63 antibodies to reveal K63-ubiquitinated forms of IMD. Cell lysates were analyzed with αV5, αMyc, or αTub to reveal the amount of, respectively, IMD-V5, dUSP36-Myc, and Tubulin, which serves as an internal loading control. Expressing dUSP36 but not dUSP36C*H* reduced the amount of K63-ubiquitinated IMD and the total amount of nonubiquitinated IMD. In a second set of samples (right panels), proteasomal degradation of proteins was blocked by MG132. Cell extracts were similarly immunoprecipitated with anti-V5 antibodies and analyzed with anti-K48 antibodies, revealing K48-ubiquitinated forms of IMD at sizes similar to K63-ubiquitinated forms. The total amount of IMD stays unchanged in dUSP36-expressing cells treated with MG132.

expression of the catalytically dead mutant, decreased the amount of K63-ubiquitinated IMD and of full-length IMD both in vivo and in S2 cells. Since treating S2 cells with a proteasome inhibitor restored a normal amount of IMD and enhanced the amount of K48-ubiquitinated forms of IMD, it is likely that IMD, like the human scaffolding protein RIP1, is subjected to both K48- and K63-linked ubiquitination and that dUSP36 indirectly promotes K48-linked polyubiquitination and degradation of IMD by hydrolyzing K63-polyubiquitin chains (Figure 7). The fact that *dUsp36* loss of function provokes a constitutive deregulation of the IMD pathway argues in favor of the existence of a steady state of the IMD pathway that is maintained at a very low basal level through the ubiquitination/deubiquitination reversible chemical modification, despite the presence of environmental microbes (Figure 7). Interestingly, IMD is K63-ubiquitinated in vivo upon infection by the DIAP2 ubiquitin ligase (N. Silverman, personal communication), reinforcing a model where IMD activation depends on its ubiquitinated status.

In a scheme where dUSP36 would be required to buffer the pathway by preventing constitutive K63-linked ubiquitination of IMD, it remains to be determined how the activity of dUSP36 is itself regulated. Whereas a constant basal level of dUSP36 might insure a permanent negative regulation of the pathway, a specific signal might block dUSP36 activity during infection. As described for the human USP36 protein (Kim et al., 2005), the dUSP36 sequence presents PEST domains (data not shown), which are characteristic for unstable proteins and might drive transient dUSP36 degradation upon infection. The hypothesis that dUSP36 inhibition might be achieved at the protein level is supported by the fact that we did not detect major transcriptional changes of *dUsp36* transcript upon infection (data not shown). In contrast, several data indicate that the mouse homolog DUB-1

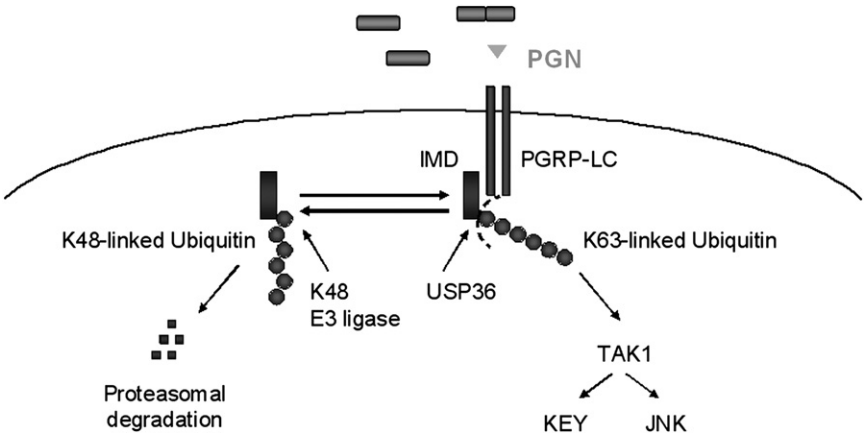

Figure 7. Proposed Model for Ubiquitin-Dependent Regulation of IMD

Linkage of K63-polyUb chains on IMD would promote signaling to TAK1, whereas linkage of K48-linked polyubiquitin chains on IMD would promote its degradation. The IMD pathway would be permanently activated by environmental bacteria and inhibited by dUSP36 via the hydrolysis of K63-polyUb chains linked to IMD. This removal would favor K48 polyubiquitination of IMD, then promoting its proteasomal-dependent degradation.

gene expression is upregulated by inflammatory cytokines (Baek et al., 2004; Zhu et al., 1996).

The IMD pathway is negatively regulated at many levels, showing the importance of preventing its constitutive activation for fly fitness. The amidase activity of some secreted PGRPs digest activating peptidoglycan (Bischoff et al., 2006; Zaidman-Rémy et al., 2006), PGRP-LF prevents PGRP-LC activation at the cell membrane (Maillet et al., 2008; Persson et al., 2007), and negative feedback loops or specific negative regulators act at the intracellular level, including the ubiquitin ligase POSH inducing TAK1 ubiquitination and degradation (Gordon et al., 2008; Tsuda et al., 2005). Three independent studies of the negative regulator PIRK pointed out that negative regulation of the IMD pathway at the level of the complex formed by PGRP-LC and IMD is a crucial step in the negative control of signal transduction (Aggarwal et al., 2008; Kleino et al., 2008; Lhocine et al., 2008). However, despite the identification of ubiquitin ligases promoting IMD activation (Zhou et al., 2005), evidence for IMD ubiquitination was lacking, and ubiquitin proteases acting on upstream elements of the IMD pathway still remained to be identified (Tsichritzis et al., 2007). Our study demonstrates that IMD is ubiquitinated by either K63-linked or K48-linked polyubiquitin chains. Furthermore, it identifies dUSP36 as a deubiquitinating enzyme involved in the negative control of innate immune signaling via IMD deubiquitination.

EXPERIMENTAL PROCEDURES

Fly Strains, Screening, and Infections

Flies were raised on standard culture medium at 25°C except if otherwise indicated. We screened a subset of 120 P{Mae-UAS.6.11}UY insertions selected for their modified stress response (Monnier et al., 2002). UY lines were crossed with the daGal4 driver line, and the progeny were infected by septic injury with a diluted exponential-phase culture of *P. aeruginosa* (Fauvarque et al., 2002) to select more resistant or sensitive lines. A second readout assay was conducted by analyzing *Dipt* expression upon infection by Gram-negative bacteria *E. coli*, *A. tumefaciens*, or *E. cloacae*.

Mapping the UY1507 insertion was performed by genomic PCR. Jumpout of the UY1507 element was conducted following standard procedure (Robertson et al., 1988). One lethal deletion was recovered among 160 independent excised lines analyzed and sequenced by PCR amplification.

Transgenic lines were constructed in the P{UAST} vector (Brand and Perrimon, 1993). P{UAS-*dUsp36*} contains the *CG-5505-RB* cDNA subcloned from LD40339 (Drosophila Genomics Resource Center). P{UAS-*dUsp36-IR*} expresses *dUsp36* cDNA sequences cloned in inverted repeats designed from the Drosophila RNAi Screening Center (http://flyrnai.org) (Flockhart et al., 2006). P{UAS-*Imd-IR*} was recovered from the VDRC (#9253) (Dietzl et al., 2007). Other stocks were recovered from the *Drosophila* Bloomington stock center.

HS-driven expression of transgenes was achieved by crossing flies with the HspGal4 (HSGal4) driver line, and progeny were submitted to sequential temperature changes: 30 min, 37°C; 30 min, 18°C; 30 min, 37°C; 1 hr, 28°C; 16 hr, 25°C.

Axenic flies were obtained as described in Brummel et al., 2004.

Northern Blots and Quantitative RT-PCR Analysis

Total RNAs were extracted from adult flies using RNA^{plus} from QBiogene (Illkirch, France). Northern blots were probed with a ^{32}P-labeled fragment of *dUsp36*, *Dipt*, or *Act* cDNA. For quantitative RT-PCR analysis, cDNAs were synthesized with AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Amsterdam). An amount of cDNA equivalent to 0.01 μg of total RNA was subjected to 40 cycles of PCR amplification consisting of a 10 s incubation at 95°C and 30 s at 60°C. Output was monitored using SYBR Green core reagents and the MX3000P instrument (Stratagene). All the results were normalized to the *RpL32* RNA level. The primer sequences were designed using PrimerQuest (http://eu.idtdna.com/Scitools/Applications/Primerquest/) and are available on request.

Construction of a Catalytic Mutant of dUSP36 and In Vitro Deubiquitinating Assays

The cysteine and histidine residues critical for catalysis at positions 111 and 369, respectively, were mutated using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) to obtain dUSP36C111S,H369N (indicated as dUSP36C*H* throughout the manuscript). In vitro deubiquitinating assays were performed following standard protocols (see Supplemental Experimental Procedures).

Immunoprecipitation and Immunoblotting

CoIP was performed in S2-cotransfected cells with 10 μg of Myc-tagged full-length *dUsp36* construct in pAc/HisB vector (Invitrogen; Cergy Pontoise, France) (dUSP36-Myc) and 10 μg of V5-tagged full-length of IMD, PGRP-LC (Choe et al., 2005), or KEY (Tsichritzis et al., 2007). Pull-down assays were performed in S2 cells transfected with the IMD-V5 construct and lysed after 48 hr. The lysate was employed in a GST-dUSP36 N-terminal or C-terminal pull-down assay. The pull-downs were blotted with antibody against V5 to detect bound IMD. To assess the enzymatic activity of dUSP36 on ubiquitinated forms of IMD, we coexpressed 2 μg of *pAc-Imd-V5* with 7 μg of empty *pAc*, 7 μg of *pAc-dUsp36-Myc*, or *pAc-dUsp36C*H*-Myc*. To block K48-linked ubiquitin chain-mediated degradation of proteins by the proteasome, MG132 was added to cell culture at 20 μM final concentrations for 4 hr. Lysates were immunoprecipitated with anti-V5 antibody and revealed with either anti-K63 or anti-K48 antibodies (Newton et al., 2008) provided by Genentech (San

Francisco). Western blots with whole-cell lysates were revealed with anti-V5 to detect IMD-V5, anti-Myc to detect dUSP36-Myc or dUSP36C*H*-Myc, or anti-Tubulin (internal loading control).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and six figures and can be found online at http://www.cell.com/cell-host-microbe/supplemental/S1931-3128(09)00316-3.

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